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14. ABSTRACT
We succeeded in completing our objectives for this research project. These accomplishments include the following: (a) synthesis and development of a flexible organophosphate substrate analog library for each of the stereoisomers of GB, GD, and VX. These compound have been utilized in the high throughput screening of mutant protein libraries; (b) site-directed mutagenesis of each amino acid within the active site of the bacterial phosphotriesterase; (c) development of mutagenic protocols for the efficient construction of protein libraries with altered catalytic properties; (d) development of high throughput spectrophotometric screens for characterization of individual mutant proteins derived from the enzyme libraries.

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FINAL REPORT

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PRINCIPAL INVESTIGATOR: Dr. Frank M. Raushel

INSTITUTION: Texas A&M University

GRANT TITLE: Enzymatic Detoxification of Chemical Warfare Agents

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OBJECTIVE: The primary objective of this research effort was to evolve and enhance the catalytic properties of the bacterial enzyme phosphotriesterase (PTE) toward the decontamination, destruction, and detection of G- and V-type nerve agents and associated analogs. We demonstrated that this same enzyme will hydrolyze G- and V-type nerve agents. The active site of the wild type phosphotriesterase was re-engineered through combinatorial mutagenesis to create libraries of mutant enzymes with altered catalytic properties. From these libraries we selected mutant proteins that have enhanced catalytic activity towards a variety of CW-agents.

APPROACH: This research program was focused on the development of a versatile enzyme-based system that was fully optimized for the decontamination, destruction, and detection of known chemical warfare agents. The catalytic properties of the wild-type enzyme phosphotriesterase (PTE) was enhanced and broadened for the hydrolytic detoxification and detection of the G- and V-type nerve agents and their associated analogs. This goal was being achieved by the reorganization of the active site of the wild type phosphotriesterase through combinatorial and directed-mutagenesis to create libraries of mutant enzymes with altered catalytic properties. High throughput screening methods were being developed to permit the isolation of those mutant proteins with improved catalytic properties toward the hydrolysis of all biologically toxic stereoisomers of the G- and V-type agents. We sought to identify and characterize the individual amino acid residues within the active site of PTE that dictate the substrate and catalytic efficiency of this enzyme.

ACCOMPLISHMENTS: We succeeded in completing our objectives for this research project. These accomplishments include the following: (a) synthesis and development of a flexible organophosphate substrate analog library for each of the stereoisomers of GB, GD, and VX. These compound have been utilized in the high throughput screening of mutant protein libraries; (b) site-directed mutagenesis of each amino acid within the active site of the bacterial phosphotriesterase; (c) development of mutagenic protocols for the efficient construction of protein libraries with altered catalytic properties; (d) development of high throughput spectrophotometric screens for characterization of individual mutant proteins derived from the enzyme libraries.

Substrate libraries. We developed chemical and enzymatic synthetic routes to the construction of organophosphate analogs that mimic the stereochemical forms of sarin

(GB), soman (GD), GF and VX. These substrate analogs were utilized in the screening and characterization of our expanding library of mutant phosphotriesterase enzymes.

High Throughput Screen. We developed a rapid and convenient method that utilizes a simple 96 and/or 384-well plate reader attached to a Beckman BioMek 2000 liquid handling system to rapidly assess the catalytic properties of the novel mutants of PTE. The method uses ultraviolet and visible spectrophotometry to detect the hydrolysis of substrate analogs with either a *p*-nitrophenol or alkyl thiol as the leaving group. The relative activities of thousands of novel mutants have been measured simultaneously without the need for protein purification.

Protein Mutagenesis. We succeeded in the preparation of all single site mutations to those 14 amino acids that are within the active site of PTE. We used the substrate library to establish the structural determinants for the stereochemical and substrate specificity of the wild type PTE. We used this information to construct combinations of site directed mutants so that we can manipulate the stereochemical and substrate specificity of PTE in a programmed direction.

Mutagenic Libraries. We succeeded in the construction of an artificial gene for the bacterial phosphotriesterase to facilitate the preparation of controlled mutant libraries of PTE. The cassette mutagenesis and PCR based systems have been merged to facilitate the preparation of thousands of mutants in a single experiment. We concentrated our efforts toward the construction of mutant enzymes that can hydrolyze the most toxic forms of the nerve agents GB, GD, GF, and VX. The power of this procedure can be illustrated with the following example. It has been determined by others that the S_P-stereoisomers of sarin and soman are significantly more toxic than are the R_P-stereoisomers. We have taken our substrate analog for the S_PS_C-isomer of soman and tested it with an array of mutant enzymes where residues H254 and H257 were randomized to all possible combination of residues (400-member library). In the rapid screen of this mutant library we found a single mutant (H254G/H257W) where the rate of hydrolysis was enhanced by about 100-fold! We then took this mutant and randomized L303 (20-mutant library). We identified L303T within the H254G/H257W parent as a substitution that had a greatly enhanced rate of turnover (~10-fold). Thus we have succeeded in the construction of a mutant (H254GH257W/L303T) that had a 1000-fold enhancement in the rate of enzymatic hydrolysis.

Synthesis of Organophosphate Analog for Genetic Selection. We succeeded in the design and synthesis of a novel organophosphate substrate analog that contains a cysteine ligand attached to the phosphorus core through the thiolate side chain. This substrate analog was used to genetically select for PTE mutants that are enhanced in the ability to hydrolyze a P-S bond. The use of an *E. coli* mutant cell line that is auxotrophic for cysteine will enable us to select for PTE mutants that require the rapid hydrolysis of this analog for growth.

CW-agent Hydrolysis. We demonstrated that mutants of phosphotriesterase can be created that have an enhanced ability to catalyze the decontamination of chemical warfare agents. In collaboration with Drs. Joseph DeFrank and Steven Harvey at Edgewood we have taken our mutant enzymes from our analog screens and tested the most promising candidates as catalysts for the detoxification of sarin, soman and VX.

These experiments have been very successful. The mutant I106A/H257Y has a turnover number for the racemic sarin of 1600 s^{-1}. To our knowledge this is the highest turnover number ever reported for enzymatic hydrolysis of sarin. The mutant G60A has a k_{cat} for the racemic form of soman of 18 s^{-1}. The mutant H254A has a turnover number for VX of >1 s^{-1}. This is higher than any published value for the hydrolysis of VX by any enzyme.

CONCLUSIONS: We demonstrated that mutants of PTE can be constructed that are significantly enhanced toward to hydrolysis of the CW-agents GB, GD, GF, and VX.

SIGNIFICANCE: The use of enzymes for the purpose of decontamination and protection of chemical warfare agents offers the advantage of a nontoxic, non-corrosive, and environmentally safe techniques. Enzyme-based systems also have the potential to significantly reduce the logistical burden. An enzyme capable of detecting G- and V-type nerve agents can be incorporated into devices for the detection of these compounds at very low concentrations. Active enzyme could be incorporated into filters, gas masks, and impregnated into protective clothing for the individual warfighter.

PATENT INFORMATION: None

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PUBLICATIONS and ABSTRACTS (for total period of grant):

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